# Not Very Funny: How a Single Mutation Causes Heritable Bradycardia

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HCN channels and their modulation by cAMP play a key role in cardiac pacemaking. In this issue of *Structure*, Xu and colleagues reveal that an arrhythmia-causing mutation of an HCN channel weakens cAMP binding to the channel by altering the local structure of its entry-exit pathway.

In the heart and nervous system, there are populations of cells that undergo autonomous rhythmic depolarization and hyperpolarization and action potential firing. These so-called pacemaker cells drive heart beat, respiration, circadian rhythms, and other physiological functions. Many ionic currents are implicated in cardiac pacemaking. One of the most important players is the "funny" current ($I_f$), also called the "queer" or "hyperpolarization-activated" current ($I_q$ or $I_h$, respectively). Genetic mutations in the genes encoding $I_f$ channels cause inherited arrhythmia (Baruscotti et al., 2010). In this issue of *Structure,* Xu et al. (2012) used an amalgam of approaches including biochemistry, electrophysiology, patch-clamp fluorometry, and X-ray crystallography to investigate the molecular mechanism of a disease-causing $I_f$ channel mutation. In doing so, this study provides an example of how diverse experimental methods are employed to address fundamental questions of protein structure-function relationships in both equilibrium and dynamic conditions and on both local and global scales.

$I_f$ was first reported in the late 1970s as a depolarizing current in the synoatrial node of heart (Brown et al., 1979) and was named "funny" because of a striking feature; unlike most voltage-gated currents, $I_f$ is activated upon hyperpolarization instead of depolarization. Thus, at a typical resting membrane potential (e.g., −60 mV), $I_f$ would activate and start to depolarize the cell (Figure 1). This, in turn, opens various voltage-gated cation channels, which further depolarize the cell and lead to action potential firing. $I_f$ deactivates during the action potential, and the cell is repolarized due to the inactivation of voltage-gated $Ca^{2+}$ channels and the opening of $K^+$ channels. When the membrane potential falls back to −50 to −60 mV, $I_f$ is activated once more, restarting the cycle (Figure 1).

The molecular correlates of $I_f$ were discovered nearly two decades later. $I_f$ arises from the activity of hyperpolarization-activated cyclic nucleotide-modulated, or HCN, channels (Santoro et al., 1997, 1998; Gauss et al., 1998; Ludwig et al., 1998). There are four closely related HCN subunits (HCN1–4). Each subunit has six transmembrane segments (S1–S6) and a pore-forming loop between S5 and S6. Four HCN subunits assemble to form a functional homomeric or heteromeric channel. HCN channels are activated by hyperpolarization and are permeant to $Na^+$ and $K^+$ ions, generating inward current at negative membrane potentials.

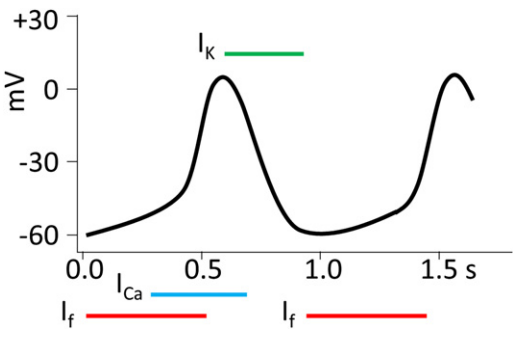


**Figure 1. The "Funny" Current $I_f$ Is Important for Cardiac Pacemaking**
$I_f$ is turned on (red line) by hyperpolarization, causing depolarization and activation of other voltage-gated cation channels including $Ca^{2+}$ channels (blue line). Deactivation of $I_f$ together with $Ca^{2+}$ channel inactivation and $K^+$ channel opening (green line) repolarize the cell back to −60 mV, restarting the cycle.

An important feature of HCN channels is that they are modulated by cAMP (DiFrancesco and Tortora, 1991). cAMP shifts the voltage dependence of channel activation to more positive voltages so that smaller hyperpolarizations can open the channels. cAMP also increases the speed and probability of channel opening. These effects partly underlie the regulation of heartbeat by the autonomic nervous system and hormones. cAMP binds to a conserved cyclic nucleotide-binding domain (CNBD) in the C terminus. CNBD is linked to S6 through a 90- to 120-amino acid linker termed the C-linker, which transmits conformational changes from the CNBD to the channel pore upon cAMP binding/unbinding (Figure 2).

The first structural insight into cAMP-mediated modulation of HCN channels came from the crystal structure of the mouse HCN2 C terminus bound to cAMP (Zagotta et al., 2003). This structure shows that the C-linker contains six α helices that participate in channel tetramerization. The CNBD is similar to that found in other cAMP-binding proteins, containing eight β sheets (β1–β8) that form a β-roll and four α helices named A, B, P, and C. cAMP is bound in a pocket formed by several β sheets and α helix C. Notably, the β4 and β5 strands engage in interactions with cAMP, and the loop between these β strands forms part of the entry-exit pathway to and from the cAMP-binding pocket (Figure 2). Interestingly, comparisons of the crystal structures of the CNBD of HCN1, HCN2, and HCN4 show that the cAMP-binding pocket itself is virtually identical among these

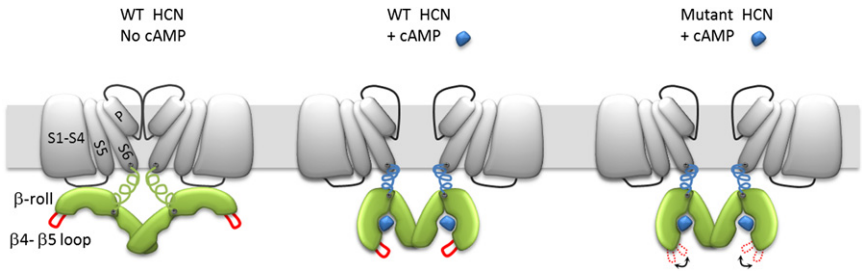


**Figure 2. The S672R Mutation Perturbs the cAMP Entry-Exit Pathway**
The schematics depict WT and S672R mutant HCN4 channels without or with bound cAMP. Two of the four subunits are shown. The C terminus of each subunit contains a CNBD (green), which is linked to S6 via the C-linker (spring). The β4-β5 loop (red), protruding from the β-roll region of the CNBD, forms the cAMP entry-exit pathway and is made more flexible by the S672R mutation. Modified from Wainger et al. (2001).

channels, but the β4-β5 loop adopts different structures (Lolicato et al., 2011).

Several HCN4 mutations have been linked to bradycardia or slowed heart rate (Baruscotti et al., 2010). The first HCN4 mutation to be unequivocally identified as a direct cause of arrhythmia was S672R, which was identified in several members of an Italian family with bradycardia (Milanesi et al., 2006). Residue S672 is located in the core of HCN4 CNBD and near but not in the cAMP-binding pocket. Initial studies showed that the S672R mutation caused a small hyperpolarizing shift of HCN channel activation but did not change the sensitivity to cAMP (Milanesi et al., 2006), seemingly consistent with the lack of a direct contact between S672 and cAMP.

The study by Xu et al. (2012) first examined steady-state binding of cAMP to an isolated HCN4 C-linker-CNBD fragment (called CL-CNBD) by using two different equilibrium binding assays, isothermal titration calorimetry, and fluorescence anisotropy. A 3- to 10-fold decrease in cAMP binding affinity was observed. The authors then examined the sensitivity of full length S672R channels to cAMP by studying a chimeric channel named mHCN2-h4 in inside-out patch-clamp recordings. In this chimera, CL-CNBD of human HCN4 was swapped into mouse HCN2, as HCN4 itself expresses poorly in *Xenopus* oocytes. The mHCN2-h4 channel reproduced key features of cAMP modulation of HCN4 channels. In contrast to previous findings (Milanesi et al., 2006), Xu et al. (2012) found the S672R mutation markedly reduced cAMP binding to the mHCN2-h4 channel (by 50-fold). The discrepancy between these studies probably lies not in the different channel types studied but in the different experimental protocols used to obtain current-voltage curves.

How does the S672R mutation reduce cAMP binding to the CNBD? To address this question, Xu et al. (2012) obtained and compared the crystal structures of wild-type and mutant HCN4 CL-CNBD bound to cAMP. The S672R mutation did not alter the global structure of CL-CNBD or the cAMP-binding pocket. Instead, it caused only local structural perturbations in the cAMP entry/exit pathway; specifically, the β4-β5 loop became disordered and more flexible (Figure 2).

Xu et al. (2012) went on to investigate dynamic, channel activity-dependent interactions between cAMP and WT and S672R mutant HCN channels. This was accomplished by using patch-clamp fluorometry, a technique that allows simultaneous recordings of channel activity and cAMP binding in isolated cell membranes. The results show that for both WT and mutant channels, cAMP binding increased greatly during channel opening, but cAMP unbinding upon channel closure was twice faster for the mutant channel than for the WT channel. Moreover, the S672R mutation reduced cAMP binding in the resting state. It is postulated that local conformational changes in the β4-β5 loop induced by the S672R mutation lead to a faster escape of cAMP from its binding site and a lower binding affinity (Figure 2).

In summary, the results of Xu et al. (2012) suggest that the S672R mutation reduces HCN4 channel currents by attenuating cAMP binding to the channel. This study highlights the notion that elements on the ligand entry-exit pathway could play a great role in ligand binding and retention, enriching our understanding of protein conformational changes that underlie the general process of ligand activation/modulation of proteins. The significance of this study is amplified by its implication for the development of molecules that specifically target the β4-β5 loop of different HCN channels.

## ACKNOWLEDGMENTS

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